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Metabolism of lysine in α-aminoadipic semialdehyde dehydrogenase-deficient fibroblasts: Evidence for an alternative pathway of pipecolic acid formation

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ABSTRACT

The mammalian degradation of lysine is believed to proceed via two distinct routes, the saccharopine and the pipecolic acid routes, that ultimately converge at the level of α-aminoadipic semialdehyde (α-AASA). α-AASA dehydrogenase-deficient fibroblasts were grown in cell culture medium supplemented with either L-[α-^{15}N]lysine or L-[ε-^{15}N]lysine to explore the exact route of lysine degradation. L-[α-^{15}N]lysine was catabolised into [^{15}N]saccharopine, [^{15}N]α-AASA, [^{15}N]Δ^1-piperideine-6-carboxylate, and surprisingly in [^{15}N]pipecolic acid, whereas L-[ε-^{15}N]lysine resulted only in the formation of [^{15}N]saccharopine. These results imply that lysine is exclusively degraded in fibroblasts via the saccharopine branch, and pipecolic acid originates from an alternative precursor. We hypothesize that pipecolic acid derives from Δ^1-piperideine-6-carboxylate by the action of Δ^1-pyrroline-5-carboxylic acid reductase, an enzyme involved in proline metabolism.

1. Introduction

In mammals, L-lysine (an essential α,ε-dibasic amino acid) is catabolised by distinct yet convergent branches. In what is believed to be the main catabolic route [1–3], L-lysine is converted into saccharopine by the action of the bifunctional enzyme α-aminoadipic semialdehyde synthase (AASS) (Fig. 1). For this reaction 2-ketoglutaric acid is a mandatory co-substrate. Saccharopine is further metabolized by AASS into α-aminoadipic semialdehyde (α-AASA) in a reaction in which glutamic acid is produced. The net result of this two-step metabolic process is the ε-deamination of L-lysine. Alternatively, L-lysine is metabolized via an α-deamination yielding 2-keto-6-aminocaproic acid, which is in equilibrium with its cyclic form Δ^1-piperideine-2-carboxylate. The latter is converted into L-pipecolic acid which is metabolized by pipecolic acid oxidase to Δ^1-piperideine-6-carboxylate, which is in equilibrium with α-AASA. At this stage the two catabolic routes converge, and α-AASA is converted to α-aminoadipic acid (α-AAA) by α-aminoadipic semialdehyde dehydrogenase, also named Antiquitin.

Current knowledge of mammalian L-lysine catabolism is based upon multiple studies in different species in which [^{14}C] labeled lysine was used as tracer [4,5]. The use of [^{14}C] labeled L-lysine in these experiments facilitated the use of liquid chromatography coupled to scintillation spectrophotometry, facilitating the detection of downstream of [^{14}C] labeled metabolites. However, a major drawback of the use of [^{14}C] labeled L-lysine is the lack of specific information characterizing specific degradation pathways, thus hampering result interpretation. Recently, the genetic defect for the majority of patients with pyridoxine dependent epilepsy (PDE) was attributed to mutations in the *Antiquitin* gene which results in α-AASA dehydrogenase deficiency, making PDE a disorder in L-lysine catabolism [6]. α-AASA dehydrogenase deficiency results in the accumulation of pathognomonic α-AASA (in cerebrospinal fluid (CSF), plasma and urine) and pipecolic acid (CSF and plasma) in affected patients [7–9]. Pilot experiments using fibroblasts derived from patients with α-AASA dehydrogenase deficiency verified that the defect is also expressed in these cells, based upon the accumulation of α-AASA and decreased formation of α-AAA. Accordingly, cultured fibroblasts represent a model system in which there is a metabolic block at the level where the two branches of lysine catabolism converge, making this cell type a useful system in which to study L-lysine metabolism using sensitive GC–MS and LC–MS/MS and stable-isotope methods available in our laboratory.

In the current study, we sought to discriminate the labeling pattern of the two branches of L-lysine catabolism using α-AASA dehydrogenase-deficient fibroblasts cell lines. This was achieved using

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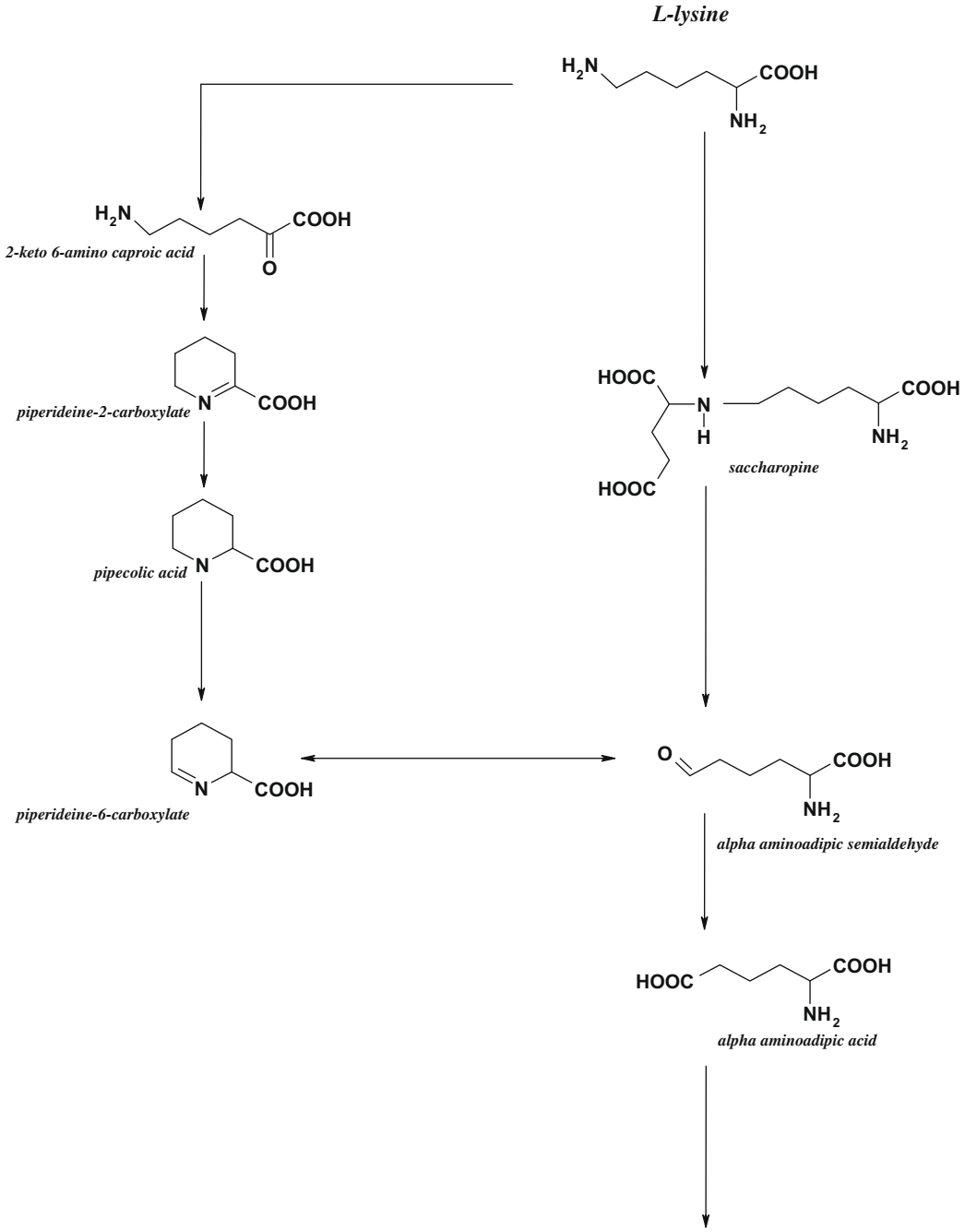

Fig. 1. Currently accepted metabolic pathway for the conversion of L-lysine in mammals, depicted to the level of AAA.

two forms of stable isotope labeled L-lysine, i.e. L-[α-^{15}N]lysine and L-[ε-^{15}N]lysine. Theoretically, incubation experiments performed with L-[α-^{15}N]lysine yield [^{15}N] incorporation in saccharopine, α-AASA, P6C and α-AAA should lysine metabolize via the saccharopine pathway. Conversely, incubation experiments with L-[ε-^{15}N]lysine should yield [^{15}N] incorporation into saccharopine, but not α-AASA and α-AAA, if the precursor is metabolized via the saccharopine pathway.

Metabolism of L-[ε-^{15}N]lysine via the pipecolic acid pathway route should yield [^{15}N] incorporation into pipecolic acid, P6C, α-AASA and α-AAA but not in saccharopine. Based upon these hypotheses, the current metabolic investigations were undertaken.

2. Materials and methods

2.1. Chemicals

Stable isotope labeled L-[α-^{15}N]lysine (95–99% purity), L-[ε-^{15}N]lysine (98% purity), and L-[U-^{13}C]lysine (98% purity) were purchased from Cambridge Isotope Laboratories, (Andover, MA, USA). The exact location of the [^{15}N] labeling in L-[α-^{15}N]lysine and L-[ε-^{15}N]lysine was verified using L-[α-^{15}N]lysine and L-[ε-^{15}N]lysine stock solutions. L-[α-^{15}N]lysine and L-[ε-^{15}N]lysine were converted into their di-*N*-trifluoroacetyl-methyl derivatives and analyzed by GC–MS operating in the electron impact mode. The GC–MS spectra obtained were compared to GC–MS spectra

Table 1
MPE's (%) in the different lysine metabolites following incubation with L-[α-^{15}N]lysine. The expressed values are the average of two independent incubation experiments (Nd indicates no detectable enrichments, i.e. <5%).

Cell line	Pipecolic acid	Saccharopine	α-AASA	P6C	α-AAA
α-AASA dehydrogenase deficient I	47	40	41	51	nd
α-AASA dehydrogenase deficient II	45	41	45	48	nd
GA I	nd	39	44	48	41

Table 2
MPE's (%) in different lysine metabolites following incubation with L-[ε-^{15}N]lysine. The expressed values are the average of two independent incubation experiments (Nd indicates no detectable enrichments, i.e. <5%).

Cell line	Pipecolic acid	Saccharopine	α-AASA	P6C	α-AAA
α-AASA dehydrogenase deficient I	nd	33	nd	nd	nd
α-AASA dehydrogenase deficient II	nd	24	nd	nd	nd
GA I	nd	28	nd	nd	nd

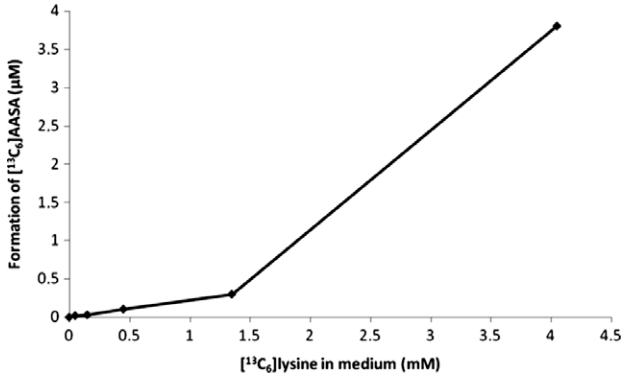

Fig. 2. Formation of [U-^{13}C$_6$]α-AASA as a function of increasing [U-^{13}C$_6$]lysine concentration.

kindly provided by the manufacturer, and were found to be identical, indicating that the two forms of [^{15}N]labeled lysine were indeed uniquely labeled in the proper nitrogen.

Lysine-free cell culture medium was prepared on request by ITK Diagnostics (Uithoorn, The Netherlands). All other chemicals and reagents used were of the highest quality available.

2.2. Fibroblast cell line and culturing procedure

Three fibroblast cell lines were employed in this study, i.e. two with α-AASA dehydrogenase deficiency and one with glutaric aciduria type I (another defect in lysine catabolism downstream of AASA dehydrogenase deficiency). Fibroblasts were cultured according to standardized protocols. For incubation experiments, fibroblasts were harvested by trypsination and few cells were transferred to a 75 cm^2 flask. The use of a small cell number significantly limited the influence of intracellular accumulation of non-labeled metabolites during the logarithmic growth phase of the cells, which would result in dilution of the isotopic enrichments. Subsequently, sterilized L-[α-^{15}N]lysine or L-[ε-^{15}N]lysine (experiments performed in duplicate for each fibroblast cell line) was added to cell cultures at a 1:1 ratio equal to the L-lysine concentration already present (i.e. 167 μM). As control, each cell line was cultured without labeled substrates. Fibroblasts were grown to confluency, after which the cell culture medium was collected and stored at −20 °C. Cells were harvested by trypsination, washed with HBSS and stored at −20 °C.

2.3. Mass isotopomer analysis of pipecolic acid, P6C, saccharopine, α-AASA, and α-AAA

Pipecolic acid was determined by GC–MS according to a modification of the procedure of Kok et al. employing negative chemical ionization mass fragmentography [10]. To 500 μL of cell culture medium, 0.01 nmol [^{2}H$_9$]pipecolic acid was added as internal standard. Pipecolic acid, [^{15}N]pipecolic acid, and [^{2}H$_9$]pipecolic acid were monitored by recording the traces of *m/z* −186, *m/z* −187 and *m/z* −195, respectively. For P6C measurement, culture medium was diluted 10× with demineralised water and 5 μL was injected onto an Xterra HPLC column (3.9 × 150 mm, 5 μM particles) using 5% MeOH/H$_2$O (containing 0.025% formic acid) as mobile phase at a flow rate of 0.8 ml/min. P6C was detected using an API 3000 MS/MS (Applied Biosystems) equipped with TurboIon electrospray operating in the positive mode. The MRM transitions *m/z* 128.1 > 82 and 129.1 > 83 were used for the detection of P6C and [^{15}N]P6C, respectively. Saccharopine, α-AASA, and α-AAA were measured by LC–MS/MS as previously described employing an FMOC derivatisation procedure [6]. For the measurements of α-AASA and α-AAA, culture medium was deproteinized using an Amicon centrifuge filter and100 μL of borate buffer (pH 10) plus 100 μL of FMOC reagent (1.5 mg/ml FMOC in acetone) were added to 50 μL of the filtrate. Subsequently, 5 μL was injected onto the LC–MS/MS system. [^{15}N]Saccharopine could not be assessed in fibroblast culture media; however, we were able to measure intracellular [^{15}N]saccharopine by the same derivatisation procedure as described for α-AASA and α-AAA using 50 μL of cell extract. The MRM transitions for saccharopine and [^{15}N]saccharopine were: *m/z* −497.2 > −257.2 and *m/z* −498.2 > −258.2; for α-AASA and [^{15}N]α-AASA: *m/z* −366.1 > −170.1 and *m/z* −367.1 > −171.1, and for α-AAA and [^{15}N]α-AAA *m/z* −382.1 > −186.1 and *m/z* −383.1 > −187.1. In all analyte determinations, L-[α-^{15}N]lysine and L-[ε-^{15}N]lysine stock solutions were included to investigate the possible presence of trace amounts of ^{15}N-labeled lysine degradation intermediates. ^{15}N-Enrichment was expressed as Molar Percentage Excess (MPE) by the equation:

$$\mathrm{MPE} = \frac{\text{corrected peak area of } {}^{15}\text{N-isotopomer}}{\text{corrected peak area of } {}^{15}\text{N-isotopomer} + \text{peak area non-labeled analyte}} * 100\% \quad (1)$$

in which the corrected peak area of ^{15}N-isotopomer indicates the correction of the peak area for the contribution of the natural occurring first isotope of the specific analyte.

3. Results

Incubation of fibroblast cell lines with L-[α-^{15}N]lysine resulted in the formation of [^{15}N]saccharopine, [^{15}N]α-AASA, and [^{15}N]P6C (Table 1). [^{15}N]Pipecolic acid was detectable in the fibroblast media of α-AASA dehydrogenase-deficient cell lines only, whereas the formation of [^{15}N]α-AAA was only detectable for the glutaric aciduria type I cell line. The observed enrichments for lysine metabolites, expressed as MPE, were close to the theoretical expected value of 50%. It is noteworthy that both AAA and pipecolic acid were present in considerable amounts in normal culture medium, and the formation of non-labeled AAA and pipecolic acid in incubation experiments with [^{15}N]labeled lysine were corrected accordingly. Incubation of cell lines with L-[ε-^{15}N]lysine resulted in formation of only [^{15}N]saccharopine, with no incorporation of [^{15}N] in α-AASA, P6C, α-AAA, and pipecolic acid (Table 2). The MPE values for [^{15}N]saccharopine were lower than would be expected.

In experiments carried out with L-[ε-^{15}N]lysine, we observed an 1.5–2-fold increase in the formation of non-labeled α-AASA with L-[ε-^{15}N]lysine incubation versus blank experiments. Additionally, experiments using L-[α-^{15}N]lysine revealed an approximately

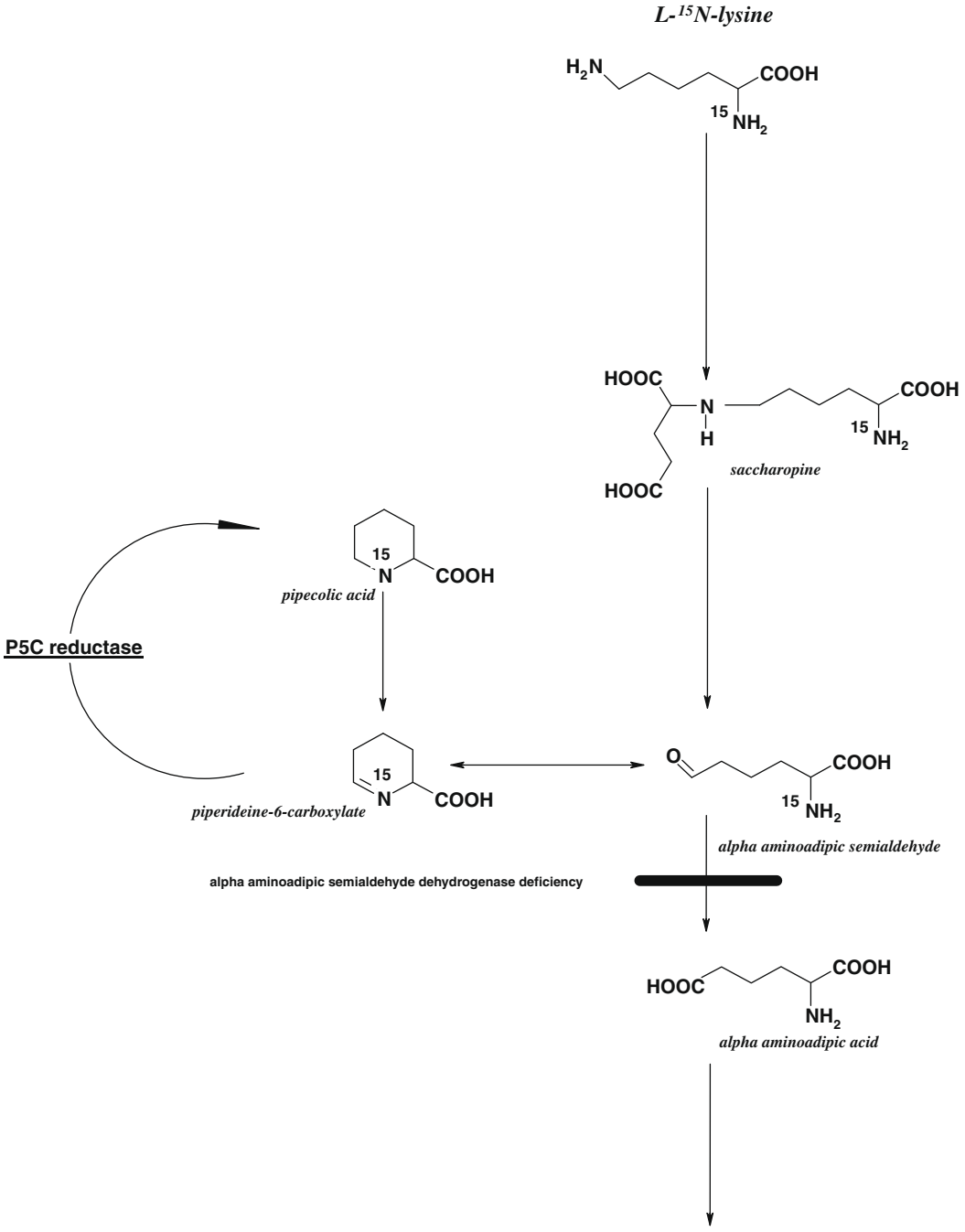

Fig. 3. Metabolism of L-[α-^{15}N]lysine in α-AASA dehydrogenase-deficient fibroblasts, showing that L-[α-^{15}N]lysine is only degraded through the saccharopine pathway. The incorporation of [^{15}N] in pipecolic acid is most likely the result of the reduction of [^{15}N]P6C by P5C reductase.

2-fold increase in the formation of total α-AASA (labeled plus non-labeled) compared to controls. Based upon these findings, we further investigated the relationship between L-lysine content of the fibroblast culture medium and the formation of α-AASA. We utilized custom made L-lysine-free fibroblast culture medium and supplemented this with increasing amounts of L-[U-$^{13}C_6$]lysine. L-[U-$^{13}C_6$]lysine was used in lieu of L-[^{15}N]lysine to allow better quantification of [U-$^{13}C_6$]-α-AASA production (no correction for the first natural isotope is required with L-[U-$^{13}C_6$]lysine). This experiment was performed using an α-AASA dehydrogenase-deficient fibroblast cell line, which was cultured as described in Section 2. [U-$^{13}C_6$]α-AASA in the culture medium was quantified relative to internal standard ([^{15}N]-α-AAA Chemotrade, Germany). The formation of [U-$^{13}C_6$]α-AASA was directly dependent upon the concentration of [U-$^{13}C_6$]lysine added to the culture medium (Fig 2). There was no significant difference in fibroblast growth over the range of 0.05–4 mM L-[U-$^{13}C_6$]lysine. However, fibroblasts grown in non-L-[U-$^{13}C_6$]lysine supplemented medium displayed retarded growth.

4. Discussion

Fibroblasts derived from individuals deficient in α-AASA dehydrogenase metabolize L-[α-^{15}N]lysine into saccharopine, α-AASA, P6C. Surprisingly, [^{15}N]pipecolic acid was also formed from L-[α-^{15}N]lysine with MPE's comparable to those of other metabolites. In contrast, when using L-[ε-^{15}N]lysine, only saccharopine was detected. Our alternate labeling strategy indicates that lysine is metabolized exclusively through the saccharopine pathway in fibroblasts, although enrichments of <5% would not be quantified. If lysine would have been at least partially metabolized via the pipecolic acid pathway, [^{15}N] incorporation would have been expected in pipecolic acid, α-AASA, P6C and α-AAA, which was not the case. Additionally, degradation of L-[α-^{15}N]lysine via the pipecolic acid pathway would not result in the formation of saccharopine, α-AASA, P6C and α-AAA, since α-deamination in the first step of lysine degradation via this reaction sequence removes the [^{15}N]label.

The formation of [^{15}N]pipecolic acid from L-[α-^{15}N]lysine is a surprising result and suggests an alternative metabolic route for the formation of pipecolic acid in fibroblasts (Fig. 3). We speculate that [^{15}N]pipecolic acid is formed from accumulated P6C by the action of pyrroline-5-carboxylate (P5C) reductase. Normally, this enzyme catalyzes the conversion of Δ^1-pyrroline-5-carboxylate to proline using NAD(P)H as cofactor. Thus far, the conversion of P6C into pipecolic acid via P5C reductase activity has not been reported in humans. In *Escherichia coli* overexpression experiments, Fujii et al. showed that P6C, derived from lysine by the action of lysine amino transferase (LAT), is converted to pipecolic acid in the presence of P5C reductase [11]. There is no available evidence for the existence of human P6C reductase. Ghadimi et al. studied [^{14}C]lysine metabolism in extracts of human and dog liver and found [^{14}C]saccharopine as the main metabolic product, with trace amounts of [^{14}C]pipecolic acid [4]. These authors concluded that lysine is preferentially metabolized via the saccharopine pathway in human liver, with metabolism via the pipecolic acid pathway as a minor metabolic route. Our hypothesis for pipecolic acid production via P5C reductase might also explain the formation of [^{14}C]pipecolic acid in the study of Ghadimi et al. [4]. Studies with [U-$^{13}C_6$]lysine revealed a direct correlation between substrate concentration and the formation of [U-$^{13}C_6$]α-AASA. Children affected with PDE require life-long pyridoxine supplementation to prevent seizures. Despite this, the long term cognitive prognosis remains poor. This may be a consequence of chronic metabolite intoxication upstream of the metabolic block, and our fibroblast results suggest that dietary lysine restriction may lower the flux through the lysine pathway, yielding a net decrease in presumably toxic intermediates.

Although our data argue against the existence of the pipecolic acid pathway of lysine metabolism in fibroblasts, we cannot extend our findings to other tissues. The literature suggests that the pipecolic acid pathway is expressed in mammalian brain, while the brain saccharopine pathway is active neonatally but declines with age [12]. Increased plasma pipecolic acid in patients with familial hyperlysinemia (AASS deficiency) supports the existence of the pipecolic acid pathway in humans [13]. However, massive elevation of lysine in these patients might give rise to nonspecific α-deamination of lysine, comparable to the process observed in hepatic methionine adenosyltransferase deficiency where supra physiological levels of methionine are α-deaminated to 2-keto-4-methylthiobutyrate [14]. The importance of the brain saccharopine pathway can also be inferred from biochemical observations in patients with L-2-hydroxyglutaric aciduria, a disorder linked to defective conversion of L-2-hydroxyglutaric acid to 2-ketoglutaric acid. These patients manifest consistent increases of CSF lysine associated with a decreased availability of 2-ketoglutaric acid, which is needed for the conversion of lysine to saccharopine [15].

Patients with α-AASA dehydrogenase deficiency also accumulate pipecolic acid in CSF, which has been attributed to the metabolic block downstream of the pipecolic acid pathway. Our results, however, suggest that increased pipecolic acid may reflect accumulation of P6C, which is converted to pipecolic acid by P5C reductase. This possible reaction sequence raises questions about the metabolic role of pipecolic acid oxidase. Since no metabolic function for pipecolic acid is known, pipecolic acid oxidase may act as an enzyme of metabolite repair [16], reconverting pipecolic acid into P6C. Future experiments in our laboratory will focus on lysine metabolism in other cell types, including hepatocytes and astrocytes, as well as leucocytes, and on further elucidation of the kinetic parameters for the conversion of P6C to pipecolic acid by P5C reductase.

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